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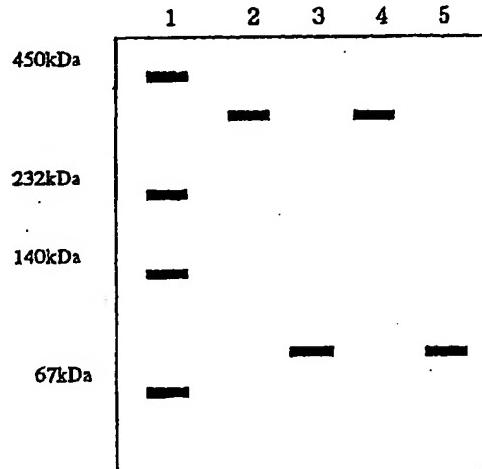
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(54) NOVEL GLUCOSE DEHYDROGENASE AND PROCESS FOR PRODUCING THE  
DEHYDROGENASE

(57) A novel glucose dehydrogenase, which is an enzyme that has high substrate specificity, can be produced at a low cost, is not affected by oxygen dissolved in a measurement sample and, in particular, has supe-

rior thermal stability is obtained by culturing a microorganism belonging to the genus *Burkholderia* and having glucose dehydrogenase producing ability in a medium and collecting glucose dehydrogenase from the medium and/or cells of the microorganism.



*Fig. 1*

DescriptionTechnical Field

5 [0001] The present invention relates to a novel glucose dehydrogenase and a method for producing the same, a DNA encoding the enzyme, a recombinant vector comprising the DNA encoding the enzyme, a transformant transformed with the recombinant vector, a novel microorganism producing the enzyme, a glucose sensor using an enzyme electrode including the enzyme, the transformant or the microorganism, and a glucose assay kit.

Background Art

[0002] Biosensors using an enzyme that specifically reacts with a particular substrate are being actively developed in various industrial fields. As for a glucose sensor, which is one of the biosensors, in particular, measurement methods and devices utilizing such methods are being actively developed mainly in medical fields.

15 [0003] The glucose sensor has a history of about 40 years since Clark and Lyons first reported about a biosensor comprising glucose oxidase and an oxygen electrode in combination in 1962 (L.c. Clark, J. and Lyons, C. "Electrode systems for continuous monitoring in cardiovascular surgery." Ann. n. y. Acad. Sci., 105: 20-45).

[0004] Thus, the adoption of glucose oxidase as an enzyme of the glucose sensor has a long history. This is because glucose oxidase shows high substrate specificity for glucose and superior thermal stability, this enzyme can further be produced in a large scale, and its production cost is lower than those of other enzymes.

20 [0005] The high substrate specificity means that this enzyme does not react with a saccharide other than glucose, and this leads to an advantage that accurate measurement can be achieved without error in measurement values.

[0006] Further, the superior thermal stability means that problems concerning denaturation of the enzyme and inactivation of its enzymatic activity due to heat can be prevented, and this leads to an advantage that accurate measurement can be performed over a long period of time.

25 [0007] However, although glucose oxidase has high substrate specificity and superior thermal stability and can be produced at a low cost, it has a problem that the enzyme is affected by dissolved oxygen as described below and this affects measurement results.

[0008] Meanwhile, in addition to glucose oxidase, a glucose sensor utilizing glucose dehydrogenase has also been developed. This enzyme is also found in microorganisms.

30 [0009] For example, there are known glucose dehydrogenase derived from *Bacillus* bacteria (EC 1.1.1.47) and glucose dehydrogenase derived from *Cryptococcus* bacteria (EC 1.1.1.119).

[0010] The former glucose dehydrogenase (EC 1.1.1.47) is an enzyme that catalyzes a reaction of  $\beta$ -D-glucose + NAD(P)+ D- $\delta$ -gluconolactone + NAD(P)H + H<sup>+</sup>, and the latter glucose dehydrogenase (EC1.1.1.119) is an enzyme that catalyzes a reaction of D-glucose + NADP+ D- $\delta$ -gluconolactone + NADPH + H<sup>+</sup>. The aforementioned glucose dehydrogenases derived from microorganisms are already marketed.

35 [0011] These glucose dehydrogenases have an advantage that they are not affected by oxygen dissolved in a measurement sample. This leads to an advantage that accurate measurement can be achieved without causing errors in measurement results even when the measurement is performed in an environment in which the oxygen partial pressure is low, or a high-concentration sample requiring a large amount of oxygen is used for the measurement.

40 [0012] However, although glucose dehydrogenase is not affected by dissolved oxygen, it has problems of poor thermal stability and substrate specificity poorer than that of glucose oxidase.

[0013] Therefore, an enzyme that overcomes disadvantages of both of glucose oxidase and glucose dehydrogenase has been desired.

45 [0014] The Inventors of the present invention reported results of their studies about glucose dehydrogenase using samples collected from soil near hot springs in Sode K., Tsugawa W., Yamazaki T., Watanabe M., Ogasawara N., and Tanaka M., Enzyme Microb. Technol., 19, 82-85 (1996); Yamazaki T., Tsugawa W. and Sode K., Appl. Biochem. and Biotec., 77-79/0325 (1999); and Yamazaki T., Tsugawa W. and Sode K., Biotec. Lett., 21, 199-202 (1999).

50 [0015] However, a bacterial strain having oxygen-producing ability had not been identified at the stage of these studies.

Disclosure of the Invention

55 [0016] An object of the present invention is to provide an enzyme that overcomes the disadvantages of both of known glucose oxidase and glucose dehydrogenase, i.e., an enzyme that shows high substrate specificity and superior thermal stability, can be produced at a low cost and is not affected by oxygen dissolved in a measurement sample.

[0017] Further, another object of the present invention is to provide a method for producing the aforementioned enzyme, a protein utilizing characteristics of the enzyme and a novel microorganism producing the enzyme.

[0018] A further object of the present invention is to provide a DNA encoding the aforementioned enzyme, a recombinant vector containing the DNA encoding the enzyme and a transformant transformed with the recombinant vector.

[0019] A still further object of the present invention is to provide a glucose sensor using an enzyme electrode including the aforementioned enzyme, transformant or microorganism and a glucose assay kit including the aforementioned enzyme.

[0020] The inventors of the present invention successfully isolated *Burkholderia cepacia* producing an enzyme achieving the aforementioned objects from soil near hot springs, and thus accomplished the present invention.

[0021] Thus, the present invention provides the followings.

- 10 (1) A method for producing glucose dehydrogenase comprising the steps of culturing a microorganism belonging to the genus *Burkholderia* and having glucose dehydrogenase producing ability in a medium, and collecting glucose dehydrogenase from the medium and/or cells of the microorganism.
- 15 (2) The method for producing glucose dehydrogenase according to (1), wherein the microorganism is *Burkholderia cepacia*.
- 15 (3) The method for producing glucose dehydrogenase according to (1) or (2), wherein the glucose dehydrogenase has the following properties:
  - 20 (i) the enzyme has an action of catalyzing dehydrogenation reaction of glucose;
  - (ii) the enzyme consists of subunits showing a molecular weight of about 60 kDa and a molecular weight of about 43 kDa in SDS-polyacrylamide gel electrophoresis under a reducing condition;
  - (iii) the enzyme shows a molecular weight of about 380 kDa in gel filtration chromatography using TSK Gel G3000SW (Tosoh Corporation); and
  - (iv) the enzyme shows an optimal reaction temperature around 45°C (Tris-HCl buffer, pH 8.0).
- 25 (4) The method for producing glucose dehydrogenase according to (3), wherein the subunit showing a molecular weight of about 43 kDa is an electron-transferring protein.
- (5) The method for producing glucose dehydrogenase according to (4), wherein the electron-transferring protein is cytochrome C.
- 30 (6) A glucose dehydrogenase, which can be produced by a microorganism belonging to the genus *Burkholderia*.
- (7) The glucose dehydrogenase according to (6), wherein the microorganism is *Burkholderia cepacia*.
- 30 (8) The glucose dehydrogenase according to (6) or (7), wherein the glucose dehydrogenase has the following properties:
  - 35 (i) the enzyme has an action of catalyzing dehydrogenation reaction of glucose;
  - (ii) the enzyme consists of subunits showing a molecular weight of about 60 kDa and a molecular weight of about 43 kDa in SDS-polyacrylamide gel electrophoresis under a reducing condition;
  - (iii) the enzyme shows a molecular weight of about 380 kDa in gel filtration chromatography using TSK Gel G3000SW (Tosoh Corporation); and
  - (iv) the enzyme shows an optimal reaction temperature around 45°C (Tris-HCl buffer, pH 8.0).
- 40 (9) The glucose dehydrogenase according to (8), wherein the subunit showing a molecular weight of about 43 kDa is an electron-transferring protein.
- (10) The glucose dehydrogenase according to (9), wherein the electron-transferring protein is cytochrome C.
- 45 (11) The glucose dehydrogenase according to any one of (8) to (10), wherein the subunit showing a molecular weight of about 60 kDa comprises the amino acid sequence of the amino acid numbers 2 to 12 in SEQ ID NO: 3.
- (12) The glucose dehydrogenase according to any one of (8) to (11), wherein the N-terminus of the subunit showing a molecular weight of 43 kDa has the amino acid sequence of SEQ ID NO: 5.
- (13) The glucose dehydrogenase according to (11), wherein the subunit showing a molecular weight of about 60 kDa is a protein defined in the following (A) or (B):
  - 50 (A) a protein which has the amino acid sequence of SEQ ID NO: 3;
  - (B) a protein which has the amino acid sequence of SEQ ID NO: 3 including substitution, deletion, insertion or addition of one or several amino acid residues and a glucose dehydrogenase activity.
- 55 (14) The glucose dehydrogenase according to (6), which shows activity peaks around 45°C and around 75°C.
- (15) A cytochrome C, which is a subunit of the glucose dehydrogenase according to (10) and has the amino acid sequence of SEQ ID NO: 5.
- (16) A DNA encoding a part of the cytochrome C according to (15) and having the nucleotide sequence of SEQ

5 ID NO: 8.

(17) A DNA encoding a part of the cytochrome C according to (15) and having the nucleotide sequence of the nucleotide numbers 2386 to 2467 in the nucleotide sequence of SEQ ID NO: 1.

(18) A DNA encoding a signal peptide of the cytochrome C according to (15) and comprising the nucleotide sequence of the nucleotide numbers 2386 to 2451 in the nucleotide sequence of SEQ ID NO: 1.

(19) A peptide which is a signal peptide of cytochrome C and has the amino acid sequence of the amino acid numbers 1 to 22 in the amino acid sequence of SEQ ID NO: 4.

(20) A protein having the following properties:

10 (i) the protein can constitute the glucose dehydrogenase according to (6) as a subunit;

(ii) the protein has a glucose dehydrogenase activity;

(iii) the protein shows a molecular weight of about 60 kDa in SDS-polyacrylamide gel electrophoresis under a reducing condition; and

(iv) the protein shows an optimal reaction temperature around 75°C (Tris-HCl buffer, pH 8.0).

15 (21) The protein according to (20), which comprises the amino acid sequence of the amino acid numbers 2 to 12 in SEQ ID NO: 3.

(22) The glucose dehydrogenase according to (21), wherein the protein is a protein defined in the following (A) or (B) defined in the following (A) or (B):

20 (A) a protein which has the amino acid sequence of SEQ ID NO: 3;

(B) a protein which has the amino acid sequence of SEQ ID NO: 3 including substitution, deletion, insertion or addition of one or several amino acid residues and a glucose dehydrogenase activity.

25 (23) A protein defined in the following (A) or (B):

(A) a protein which has the amino acid sequence of SEQ ID NO: 3;

(B) a protein which has the amino acid sequence of SEQ ID NO: 3 including substitution, deletion, insertion or addition of one or several amino acid residues and a glucose dehydrogenase activity.

30 (24) A DNA encoding a protein defined in the following (A) or (B):

(A) a protein which has the amino acid sequence of SEQ ID NO: 3;

(B) a protein which has the amino acid sequence of SEQ ID NO: 3 including substitution, deletion, insertion or addition of one or several amino acid residues and a glucose dehydrogenase activity.

35 (25) The DNA according to (24), which is a DNA defined in the following (a) or (b):

40 (a) a DNA which comprises the nucleotide sequence of the nucleotide numbers 764 to 2380 in the nucleotide sequence of SEQ ID NO: 1;

(b) a DNA which is hybridizable with a nucleotide sequence comprising the sequence of the nucleotide numbers 764 to 2380 in SEQ ID NO: 1 or a probe that can be prepared from the sequence under a stringent condition and encodes a protein having a glucose dehydrogenase activity.

45 (26) A recombinant vector comprising the DNA according to (24) or (25).

(27) The recombinant vector according to (26), which comprises nucleotide sequences encoding the signal peptide according to (18) and a β-subunit.

(28) A transformant transformed with the DNA according to (24) or (25) or the recombinant vector according to (26) or (27).

50 (29) A method for producing glucose dehydrogenase comprising the steps of culturing the transformant according to (28) to produce glucose dehydrogenase as an expression product of the DNA, and collecting it.

(30) A *Burkholderia cepacia* KS1 strain (FERM BP-7306).

(31) A glucose sensor using an enzyme electrode including the glucose dehydrogenase according to any one of (6) to (14), the protein according to any one of (20) to (23), the transformant according to (27) or the strain according to (30).

(32) A glucose assay kit including the glucose dehydrogenase according to any one of (6) to (14) or the protein according to any one of (20) to (23).

(33) A protein having the amino acid sequence of SEQ ID NO: 2.

- (34) A DNA encoding a protein having the amino acid sequence of SEQ ID NO: 2.
- (35) The DNA according to (34), which comprises the nucleotide sequence of the nucleotide numbers 258 to 761 in the nucleotide sequence of SEQ ID NO: 1.
- (36) A DNA comprising the DNA according to (34) or (35) and the DNA according to (24) or (25) in this order.
- 5 (37) The DNA according to (36), which comprises the nucleotide sequence of the nucleotide numbers 258 to 2380 in the nucleotide sequence of SEQ ID NO: 1.
- (38) A recombinant vector comprising the DNA according to (36) or (37).
- (39) The recombinant vector according to (38), which comprises nucleotide sequences encoding the signal peptide according to (18) and a β-subunit.
- 10 (40) A transformant transformed with the DNA according to (36) or (37) or the recombinant vector according to (38) or (39).
- (41) A method for producing glucose dehydrogenase comprising the steps of culturing the transformant according to (40) to produce glucose dehydrogenase as an expression substance of the DNA according to (36) or (37), and collecting it.

15

[0022] Hereafter, the present invention will be explained in detail.

<1> Novel bacterial strain producing glucose dehydrogenase of the present invention

- 20 [0023] The enzyme of the present invention (hereinafter, also referred to as "the enzyme" or "GDH") can be produced by a bacterium belonging to the genus *Burkholderia*. The *Burkholderia* bacterium used for the present invention is not particularly limited so long as it is a *Burkholderia* bacterium having ability to produce the enzyme. However, *Burkholderia cepacia*, in particular, the *Burkholderia cepacia* KS1 strain is preferred. This bacterial strain is a novel bacterial strain isolated by the inventors of the present invention from soil near hot springs as described later in the examples and was identified as *Burkholderia cepacia* based on its bacteriological properties. Conventionally, it has been unknown that a microorganism belonging to the genus *Burkholderia* can produce glucose dehydrogenase. This bacterial strain was designated as KS1 strain. This strain was deposited at International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology (Tsukuba Central 6, 1-1, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan, postal code: 305-8566) on September 25, 2000 and received a microorganism accession number of FERM BP-7306.
- 25 [0024] The Inventors of the present invention obtained some *Burkholderia cepacia* strains other than the *Burkholderia cepacia* KS1 strain, which were deposited at Institute for Fermentation (Osaka, IFO) or Japan Collection of Microorganisms (JCM), the Institute of Physical and Chemical Research, and measured their glucose dehydrogenase activities. As a result, they confirmed that all of these bacterial strains had the activity.

30

<2> Glucose dehydrogenase of the present invention

- 35 [0025] If a *Burkholderia* bacterium having glucose dehydrogenase producing ability, for example, the *Burkholderia cepacia* KS1 strain, is cultured in a nutrient medium used for usual culture of a microorganism, preferably a medium containing glucose or a substance containing glucose in order to increase the enzyme producing ability, the glucose dehydrogenase of the present invention is produced and accumulated in a culture product or cultured cells. Therefore, it can be collected by a known method. The method for producing the enzyme will be specifically explained by exemplifying the *Burkholderia cepacia* KS1 strain. First, the *Burkholderia cepacia* KS1 strain is cultured in a suitable nutrient medium, for example, a medium containing suitable carbon source, nitrogen source, inorganic salts, glucose or substances containing these and so forth to produce and accumulate the enzyme in the culture product or the cultured cells.
- 40 [0026] As the carbon sources, any substance that can be assimilated can be used, and examples include, for example, D-glucose, L-arabinose, D-xylose, D-mannose, starch, various peptones and so forth. As the nitrogen sources, there can be used yeast extract, malt extract, various peptones, various meat extracts, corn steep liquor, amino acid solutions and organic and inorganic nitrogen compounds such as ammonium salts or substances containing these. As the inorganic salts, there can be used various phosphoric acid salts and salts of magnesium, potassium, sodium, calcium and so forth. Further, as required, various inorganic and organic substances required for growth of the bacterium or production of the enzyme, for example, silicone oil, sesame oil, defoaming agents such as various surfactants and vitamins can be added to the medium.
- 45 [0027] As for the culture method, although either liquid culture or solid culture may be used, liquid culture is usually preferred.
- 50 [0028] The enzyme of the present invention can be obtained from the medium and/or the cells in the culture obtained as described above. The enzyme existing in the cells can be obtained as a cell extract by disrupting or lysing the cells.
- 55 [0029] The glucose dehydrogenase in the culture product or the cell extract can be purified by a suitable combination

of chromatography techniques using an ion exchanger, a gel filtration carrier, a hydrophobic carrier and so forth.

[0030] The activity of the enzyme can be measured by the same methods as known methods for measurement of the glucose dehydrogenase activity. Specifically, the activity can be measured by, for example, the method described later in the examples.

5 [0031] Physicochemical properties of the novel glucose dehydrogenase of the present invention are as follows:

- (i) the enzyme has an action of catalyzing dehydrogenation reaction of glucose;
- (ii) the enzyme consists of subunits showing a molecular weight of about 60 kDa and a molecular weight of about 43 kDa in SDS-polyacrylamide gel electrophoresis under a reducing condition;
- 10 (iii) the enzyme shows a molecular weight of about 380 kDa in gel filtration chromatography using TSK Gel G3000SW (Tosoh Corporation); and
- (iv) the enzyme shows an optimal reaction temperature around 45°C (Tris-HCl buffer, pH 8.0).

[0032] The glucose dehydrogenase shows an activity peak around 45°C under the aforementioned condition, and 15 also shows an activity peak around 75°C (refer to Fig. 3 (a)). No GDH has been known which shows the activity peak in two of temperature regions as described above.

[0033] The molecular weight and the optimal temperature can be measured by the methods described later in the examples.

[0034] The aforementioned glucose dehydrogenase of the present invention consists of two of separate polypeptides, 20 the α-subunit having a molecular weight of about 60 kDa and the β-subunit having a molecular weight of about 43 kDa (hereinafter, this glucose dehydrogenase is also referred to as "multimer enzyme"). The inventors of the present invention further investigated these two of subunits in detail.

[0035] It was found that the β-subunit was cytochrome C (as shown later in the examples). A protein containing only 25 the α-subunit exhibits the following physicochemical properties:

- (i) the protein can constitute the glucose dehydrogenase as a subunit;
- (ii) the protein has a glucose dehydrogenase activity;
- (iii) the protein shows a molecular weight of about 60 kDa in SDS-polyacrylamide gel electrophoresis under a reducing condition; and
- 30 (iv) the protein shows an optimal reaction temperature around 75°C (Tris-HCl buffer, pH 8.0).

[0036] The optimal temperature can be measured by the method described later in the examples.

[0037] Since this protein itself has the enzymatic activity, the protein may be optionally called as "peptide enzyme" or "enzyme" depending on the content of the explanation.

35 [0038] As a specific embodiment of the peptide enzyme of the present invention, a protein having the amino acid sequence of SEQ ID NO: 3 can be mentioned. Further, this peptide enzyme may be a protein having the amino acid sequence containing substitution, deletion, insertion or addition of one or more amino acid residues in the amino acid sequence of SEQ ID NO: 3 so long as it has the GDH activity. Although an amino acid sequence that can be encoded by the nucleotide sequence of SEQ ID NO: 1 is shown as SEQ ID NO: 3, the methionine residue at the N-terminus 40 may be eliminated after translation.

[0039] Further, as a specific embodiment of the multimer enzyme of the present invention, there can be mentioned a multimer containing a protein of which α-subunit has the amino acid sequence of SEQ ID NO: 3. Further, the aforementioned multimer enzyme may be a multimer containing a protein of which α-subunit has the amino acid sequence of SEQ ID NO: 3 including substitution, deletion, insertion or addition of one or more amino acid residues, so long as 45 it has the GDH activity.

[0040] In the present invention, "one or more" means a number of 1 to 10, preferably 1 to 5, particularly preferably 1 to 3.

[0041] The inventors of the present Invention confirmed existence of a γ-subunit in addition to the aforementioned α-subunit and β-subunit.

50 [0042] In the examples described later, the γ-subunit was removed at the stage of purifying the enzyme of the present invention from a culture supernatant or cell extract, and therefore the γ-subunit was not confirmed in the purified enzyme. However, as shown in the examples, when the γ-subunit was expressed together with the α-subunit, a high enzymatic activity was obtained in comparison with the case where only the α-subunit was expressed. This suggested that the γ-subunit was a protein involved in the production of the α-subunit in a microbial cell in some sort of way. Assuming that the specific activity of the α-subunit (enzymatic activity per protein) is the same in either case, a lower enzymatic activity indicates a smaller amount of the α-subunit as an enzyme since the enzymatic activity reflects the amount of the enzyme. On the other hand, the produced α-subunit may be protected by the γ-subunit in a certain manner, or although the α-subunit as a protein is fully expressed, it cannot have the three-dimensional structure for exhibiting the

enzymatic activity due to the absence of  $\gamma$ -subunit, and thus the enzymatic activity may become low. In either case, a high enzymatic activity can be obtained when the  $\gamma$ -subunit is expressed together with the  $\alpha$ -subunit.

<3> DNA of the present invention

5

[0043] The DNA of the present invention can be obtained from a microorganism containing the DNA of the present invention, for example, *Burkholderia cepacia*. The DNA of the present invention was isolated from chromosomal DNA of *Burkholderia cepacia* in the process of accomplishing the present invention. However, since its nucleotide sequence and the amino acid sequence encoded by this nucleotide sequence were elucidated by the present invention, the DNA 10 can also be obtained by chemical synthesis based on those sequences. Further, the DNA of the present invention can also be obtained from chromosomal DNA of *Burkholderia cepacia* or the like by hybridization or PCR using an oligonucleotide prepared based on the aforementioned sequences as a probe or a primer.

15

[0044] In addition to a DNA which encodes a protein having the amino acid sequence of SEQ ID NO: 3, the DNA of the present invention may be a DNA which encodes a protein having an amino acid sequence of SEQ ID NO: 3 containing substitution, deletion, insertion or addition of one or more amino acid residues in the amino acid sequence and has the GDH activity.

20

[0045] As the DNA of the present invention, there can be specifically mentioned a DNA comprising the nucleotide sequence of the nucleotide numbers 764 to 2380 in the nucleotide sequence of SEQ ID NO: 1. The nucleotide sequence of the nucleotide numbers 764 to 2380 in the nucleotide sequence of SEQ ID NO: 1 encodes the  $\alpha$ -subunit of GDH having the amino acid sequence of SEQ ID NO: 3.

[0046] Further, the DNA of the present invention may also be a DNA which is hybridizable with the nucleotide sequence of the nucleotide numbers 764 to 2380 in the nucleotide sequence of SEQ ID NO: 1 or a probe that can be prepared from the sequence under a stringent conditions and encodes a protein having the GDH activity.

25

[0047] It is estimated that the nucleotide sequence of the nucleotide numbers 258 to 761 in the nucleotide sequence of SEQ ID NO: 1 encodes the  $\gamma$ -subunit. The amino acid sequence is shown in SEQ ID NO: 2. It is considered that, since the structural gene of the  $\gamma$ -subunit is included in a region upstream from that of the  $\alpha$ -subunit, and thus the  $\gamma$ -subunit is expressed first and already exists as a protein upon the production of the  $\alpha$ -subunit by a microorganism, the  $\alpha$ -subunit can be efficiently produced in the microorganism. Therefore, the DNA of the present invention may include a DNA encoding the amino acid sequence of SEQ ID NO: 2 in addition to the aforementioned DNA.

30

[0048] A DNA encoding a protein substantially identical to the aforementioned protein having the amino acid sequence of SEQ ID NO: 3 can be obtained by, for example, a method such as the site-directed mutagenesis or mutagenesis treatment. The GDH activity of a protein encoded by a DNA introduced with a mutation can be measured, for example, as follows.

35

[0049] An enzyme sample and glucose as a substrate are added to 10 mM potassium phosphate buffer (pH 7.0) containing 594  $\mu$ M methylphenazine methosulfate (mPMS) and 5.94  $\mu$ M 2,6-dichlorophenol-indopheol (DCIP) and incubated at 37°C. Change in absorbance of the DCIP at 600 nm is monitored by using a spectrophotometer, and the absorbance decreasing rate is measured as an enzymatic reaction rate.

40

[0050] Further, the nucleotide sequence consisting of the nucleotide of the nucleotide number 2386 and the sequence after the nucleotide of the nucleotide number 2386 in the nucleotide sequence of SEQ ID NO: 1 is estimated to encode the  $\beta$ -subunit. Further, the nucleotide sequence of the nucleotide numbers 2386 to 2451 is estimated to encode the signal peptide of the  $\beta$ -subunit. An estimated amino acid sequence of this signal peptide is the amino acid sequence of amino acid numbers 1 to 22 in SEQ ID NO: 4. The signal peptide is a peptide necessary for a protein synthesized in ribosome to be secreted through the membrane and has been found to comprise 15 to 30 hydrophobic amino acid residues. Therefore, since the amount of proteins in the culture supernatant is increased due to the existence of the signal peptide, this is a peptide effectively acting in a method of producing a protein.

45

[0051] Hereafter, an example of a method for obtaining the DNA of the present invention will be explained.

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[0052] Chromosomal DNA is isolated from a microorganism such as *Burkholderia cepacia* and purified, and the chromosomal DNA is cleaved by ultrasonication, restriction enzyme treatment or the like and ligated to a linear expression vector and cyclized by using a DNA ligase or the like to construct a recombinant vector. The obtained recombinant vector is introduced into a host microorganism in which the vector is autonomously replicable, and the transformants are screened by using a vector marker and expression of an enzymatic activity as indexes to obtain a microorganism harboring a recombinant vector containing a gene encoding GDH. The recombinant vector contained in the obtained microorganism is expected to contain at least the nucleotide sequence encoding the  $\alpha$ -subunit. Further, if the cloned fragment has a sufficient size, it is very likely that the nucleotide sequence encoding the  $\gamma$ -subunit is also contained.

55

[0053] Then, the microorganism having the recombinant vector can be cultured, the recombinant vector can be isolated from the cells of the cultured microorganism and purified, and the gene encoding GDH can be collected from the expression vector. For example, chromosomal DNA serving as a gene donor is specifically collected, for example,

as follows.

[0054] The aforementioned gene donor microorganism can be cultured with stirring for 1 to 3 days, for example, and cells can be collected by centrifugation from the obtained culture broth and then lysed to prepare cell lysate containing the GDH gene. As the method for lysis of the cells, a treatment is performed by using a bacteriolytic enzyme such as 5 lysozyme, and other enzymes such as protease and surfactants such as sodium dodecylsulfate (SDS) are used in combination as required. Further, a physical cell disruption method such as freeze and thawing or French press treatment may also be employed in combination.

[0055] The DNA can be isolated and purified from the lysate obtained as described above in a conventional manner, for example, by a suitable combination of deproteinization by phenol treatment or protease treatment, ribonuclease 10 treatment, alcohol precipitation and so forth.

[0056] The DNA isolated and purified from a microorganism can be cleaved by, for example, ultrasonication, restriction enzyme treatment or the like. Preferably, a type-II restriction enzyme, which acts on a specific nucleotide sequence, is suitably used. The restriction enzyme used may generate an end matching a digested end of a vector, or the digested end may be blunt-ended by using an arbitrary restriction enzyme and ligated to the vector.

[0057] As the vector used for cloning, a phage that can autonomously grow in a host microorganism or a plasmid 15 that is constructed for gene recombination is suitable. Examples of the phage include, for example, when *Escherichia coli* is used as the host microorganism, Lambda gt10, Lambda gt11 and so forth. Further, examples of the plasmid include, for example, when *Escherichia coli* is used as the host microorganism, pBR322, pUC18, pUC118, pUC19, pUC119, pTrc99A and pBluescript as well as SuperCosI, which is a cosmid, and so forth.

[0058] Upon the cloning, a vector fragment can be obtained by digesting the aforementioned vector with a restriction 20 enzyme used for the digestion of a microbial DNA as the aforementioned donor of a gene encoding GDH. However, a restriction enzyme identical to the restriction enzyme used for the digestion of the microbial DNA does not necessarily need to be used. The method for ligating the microbial DNA fragment and the vector DNA fragment may be a known method using a DNA ligase. For example, an adhesive end of the microbial DNA fragment and an adhesive end of the 25 vector fragment are ligated, and then a recombinant vector containing the microbial DNA fragment and the vector DNA fragment is produced by using a suitable DNA ligase. If required, after the ligation, the fragment can also be introduced into the host microorganism to produce the recombinant vector by utilizing a DNA ligase existing in the microorganism.

[0059] The host microorganism used for the cloning is not particularly limited so long as the recombinant vector is stable and can autonomously grow in the host, and a foreign gene can be expressed in the host. *Escherichia coli* 30 DH5 $\alpha$ , XL-1 BlueMR and so forth can generally be used.

[0060] As the method for introducing the recombinant vector into the host microorganism, for example, when the host microorganism is *Escherichia coli*, the competent cell method using calcium treatment, electroporation or the like can be used.

[0061] Whether the cloned fragment obtained by the aforementioned method encodes GDH can be confirmed by 35 decoding the nucleotide sequence of the fragment in a conventional manner.

[0062] The DNA of the present invention can be obtained by collecting a recombinant vector from the transformant obtained as described above.

[0063] GDH can be produced by culturing a transformant containing the DNA of the present invention or a recombinant vector containing the DNA to produce GDH as an expression product of the DNA and collecting it from the cells 40 or culture broth. For this production, although the DNA of the present invention may be a DNA encoding the  $\alpha$ -subunit, the expression efficiency can be increased by further expressing the  $\gamma$ -subunit together with the  $\alpha$ -subunit.

[0064] Examples of the microorganism in which GDH is produced include enteric bacteria such as *Escherichia coli*, Gram-negative bacteria such as those of the genera *Pseudomonas* and *Gluconobacter*. Gram-positive bacteria including *Bacillus* bacteria such as *Bacillus subtilis*, yeasts such as *Saccharomyces cerevisiae* and filamentous fungi such 45 as *Aspergillus niger*. However, the microorganism is not limited to these microorganisms, and any host microorganism suitable for production of foreign proteins can be used.

[0065] The GDH gene contained in the once selected recombinant vector containing the GDH gene can be easily transferred into a recombinant vector that can be replicated in a microorganism by recovering a DNA which is the GDH gene from the recombinant vector containing the GDH gene by using a restriction enzyme or by PCR and ligating it to another vector fragment. Further, the microorganism can be easily transformed with these vectors, for example, by the competent cell method using calcium treatment for *Escherichia* bacteria, the protoplast method for *Bacillus* bacteria, the KU or KUR method for yeasts, the micromanipulation method for filamentous fungi and so forth. Further, electroporation can also be widely used.

[0066] The host microorganism into which a target recombinant vector is introduced can be selected by searching 55 a microorganism that simultaneously expresses a drug resistance marker of the vector containing the target DNA and the GDH activity. For example, a microorganism growing in a selective medium based on the drug resistance marker and producing GDH can be selected.

[0067] As for the culture method of the transformant, culture conditions can be selected by considering nutritional

and physiological properties of the host. In many cases, liquid culture is performed. It is industrially advantageous to perform aerobic culture with stirring.

[0068] As nutrients of the medium, those usually used for culture of microorganisms can be widely used. As carbon sources, any carbon compounds that can be assimilated can be used, and examples thereof include glucose, sucrose, lactose, maltose, lactose, molasses, pyruvic acid and so forth. Further, as nitrogen sources, any nitrogen compounds that can be utilized can be used, and examples thereof include peptone, meat extracts, yeast extract, casein hydrolysate, soybean meal alkaline extract and so forth. In addition, phosphoric acid salts, carbonic acid salts, sulfuric acid salts, salts of magnesium, calcium, potassium, iron, manganese, zinc and so forth, particular amino acids, particular vitamins and so forth are used as required.

[0069] Although the culture temperature can be appropriately changed in a range in which bacteria grow and produce GDH, it is preferably about 20°C to 42°C. The culture time somewhat varies depending on the conditions. However, the culture can be completed at an appropriate time estimated to give the maximum GDH level, and the culture time is usually about 12 to 72 hours. Although pH of the medium can be appropriately changed in a range in which bacteria grow and produce GDH, it is preferably in the range of about pH 6.0 to 9.0.

[0070] The culture broth containing cells producing GDH in the culture can be collected and utilized as they are. However, when GDH exists in the culture broth, the culture broth is usually separated into a GDH-containing solution and microorganism cells by filtration, centrifugation or the like in a conventional manner and then used. When GDH exists in the cells, the cells are collected from the obtained culture by means of filtration, centrifugation or the like, and then the cells are disrupted by a mechanical method or an enzymatic method such as use of lysozyme, and further added with a chelating agent such as EDTA and a surfactant to solubilize GDH, as required, to isolate and collect GDH as an aqueous solution.

[0071] GDH can be precipitated from the GDH-containing solution obtained as described above by, for example, vacuum concentration, membrane concentration, salting out with ammonium sulfate, sodium sulfate or the like, or a fractional precipitation with a hydrophilic organic solvent such as methanol, ethanol and acetone. Further, heat treatment and isoelectric point treatment are also effective purification means. Then, GDH can be purified by a suitable combination of gel filtration using an adsorbent or gel filtration agent, absorption chromatography, ion exchange chromatography and affinity chromatography to obtain purified GDH.

[0072] A purified enzyme preparation can be obtained by isolation and purification based on column chromatography. Although the purified enzyme preparation is preferably purified to such an extent that a single band should be obtained in electrophoresis (SDS-PAGE), it may contain the  $\gamma$ -subunit.

[0073] The purified enzyme obtained as described above can be made into powder by, for example, lyophilization, vacuum drying, spray drying or the like and distributed.

[0074] Further, the amino acid sequence of the  $\beta$ -subunit can also be determined in the same manner as in the determination of the amino acid sequence of the  $\alpha$ -subunit described later in the examples, and a DNA encoding the  $\beta$ -subunit can be isolated based on the sequence. Further, the  $\beta$ -subunit can also be produced by using the obtained DNA. Further, the multimer enzyme can also be produced by using a DNA encoding the  $\alpha$ -subunit and DNA encoding the  $\beta$ -subunit.

#### <4> Glucose sensor of the present invention

[0075] The glucose sensor of the present invention is characterized by using the enzyme of the present invention (the aforementioned multimer enzyme or peptide enzyme, or the aforementioned multimer enzyme or peptide enzyme containing the  $\gamma$ -subunit), the transformant of the present invention, or the microorganism of the present invention (*Burkholderia cepacia* KS1 strain) as an enzyme electrode. As the electrode, a carbon electrode, gold electrode, platinum electrode or the like can be used, and the enzyme of the present invention is immobilized on this electrode. Examples of the method for immobilization include a method of using a crosslinking reagent, a method of entrapping the enzyme in a polymer matrix, a method of covering the enzyme with a dialysis membrane, methods of using a photocrosslinking polymer, conductive polymer, oxidation-reduction polymer or the like. Alternatively, the enzyme may be immobilized in a polymer or immobilized on an electrode by adsorption together with an electronic mediator of which typical examples are ferrocene and derivatives thereof, or these methods may be used in combination. Typically, the glucose dehydrogenase of the present invention is immobilized on a carbon electrode by using glutaraldehyde, and glutaraldehyde is blocked by a treatment with a reagent having an amine group.

[0076] The glucose concentration can be measured as follows. A buffer is placed in a constant temperature cell and added with a mediator, and a constant temperature is maintained. As the mediator, potassium ferricyanide, phenazine methosulfate and so forth can be used. An electrode on which the enzyme of the present invention is immobilized is used as a working electrode, and a counter electrode (e.g., platinum electrode) and a reference electrode (e.g., Ag/AgCl electrode) are used. A constant voltage is applied to the carbon electrode, and after a steady-state current is obtained, a sample containing glucose is added and the increase of the current is measured. The glucose concentration

in the sample can be calculated according to a calibration curve produced by using glucose solutions having standard concentrations.

**<5> Glucose assay kit of the present invention**

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[0077] The saccharide assay kit of the present invention is characterized by including the enzyme of the present invention (the aforementioned multimer enzyme or peptide enzyme, or the aforementioned multimer enzyme or peptide enzyme containing the  $\gamma$ -subunit). The glucose assay kit of the present invention includes the enzyme of the present invention in an amount sufficient for at least one assay. Typically, the kit includes, in addition to the enzyme of the present invention, a buffer, a mediator, standard solutions of glucose or the like for creating a calibration curve, which are necessary for the assay, and a guideline for use. The enzyme of the present invention can be provided in various forms, for example, as a lyophilized reagent or a solution in an appropriate storage solution.

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**Brief Description of the Drawings**

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**[0078]**

Fig. 1 shows a molecular weight of the enzyme of the present invention determined by native PAGE electrophoresis.

Fig. 2 shows an electrophoretic photograph showing a molecular weight of the enzyme of the present invention based on SDS-PAGE electrophoresis.

Fig. 3 shows the optimal reaction temperature (a) and thermal stability (b) of the enzyme of the present invention.

Fig. 4 shows the optimal reaction temperature (a) and thermal stability (b) of a peptide enzyme constituting only the  $\alpha$ -subunit of the enzyme of the present invention.

Fig. 5 shows results of spectrophotometric analyses of the enzyme of the present invention in the absence or

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presence of glucose before heat treatment (a) and spectrophotometric analyses of the enzyme of the present invention in the absence or presence of glucose after heat treatment (b).

Fig. 6 shows responses of a glucose sensor using GDH obtained from a transformant to glucose at various temperatures.

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**Best Mode for Carrying out the Invention**

[0079] The present invention will be explained more specifically with reference to the following examples.

**Example 1: Acquisition of bacterium having glucose dehydrogenase producing ability**

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**[Screening]**

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[0080] The microorganism of the present invention was obtained by collecting soil near various hot springs in Japan and selecting a bacterium having a glucose dehydrogenase activity among bacteria utilizing glucose as a nutrient from the soil.

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[0081] The results of investigation of morphological characteristics, growth characteristics and physiological characteristics of this strain are shown below.

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<b>[Bacteriological characteristics]</b>	
Gram staining	negative
Cell morphology	rod-shaped
With polar flagellum	
Mobility	positive
Number of fragments	> 5
Optimal growth temperature	45°C
Oxidase	negative
Catalase	positive
Production of acetoin	negative
Production of H <sub>2</sub> S	negative
Production of indole	negative
Acid from glucose	positive

(continued)

[Bacteriological characteristics]	
5	Arginine dihydrolase negative
	Urease negative
	$\beta$ -Glucosidase negative
	Protease negative
10	$\beta$ -Galactosidase positive
	Lysine carboxylase negative
	Ornithine carboxylase negative
	Reduction of nitrate positive

[Assimilation characteristics]	
15	Glycerol positive
	Erythritol negative
	D-Arabinose negative
20	L-Arabinose positive
	Ribose positive
	D-Xylose positive
	L-Xylose negative
25	Adonitol positive
	$\beta$ -Methyl-xyloside negative
	Galactose positive
	D-Glucose positive
	D-Fructose positive
30	D-Mannose positive
	L-Sorbose negative
	Rhamnose negative
	Dulcitol positive
35	Inositol positive
	Mannitol positive
	Sorbitol positive
	$\alpha$ -Methyl-D-mannoside negative
40	$\alpha$ -Methyl-D-glucoside negative
	N-Acetyl-glucosamine positive
	Amygdaline negative
	Arbutin negative
	Esculin negative
45	Salicin negative
	Cellobiose negative
	Maltose negative
	Lactose negative
	Melibiose negative
50	Sucrose negative
	Trehalose positive
	Inulin negative
	Melezitose negative
55	D-Raffinose negative
	Amidon negative
	Glycogen negative
	Xylitol positive

(continued)

[Assimilation characteristics]		
5	$\beta$ -Gentiobiose	negative
	D-Turanose	negative
	D-Lyxose	negative
	D-Tagatose	negative
	D-Fucose	negative
10	L-Fucose	negative
	D-Arabitol	positive
	L-Arabitol	positive
	Gluconic acid	positive
	2-Ketogluconic acid	positive
15	5-Ketogluconic acid	negative
	Capric acid	positive
	Adipic acid	positive
	Malic acid	positive
	Citric acid	positive
20	Phenyl acetate	positive

[Oxidation characteristics]		
25	Glycerol	negative
	Erythritol	negative
	D-Arabinose	negative
	L-Arabinose	positive
30	Ribose	positive
	D-Xylose	positive
	L-Xylose	negative
	Adonitol	positive
	$\beta$ -Methyl-xyloside	negative
35	Galactose	positive
	D-Glucose	positive
	D-Fructose	positive
	D-Mannose	positive
40	L-Sorbose	negative
	Rhamnose	negative
	Dulcitol	positive
	Inositol	positive
	Mannitol	positive
	Sorbitol	positive
45	$\alpha$ -Methyl-D-mannoside	negative
	$\alpha$ -Methyl-D-glucoside	negative
	N-Acetyl-glucosamine	negative
	Amygdaline	negative
50	Arbutin	negative
	Esculin	positive
	Salicin	negative
	Cellobiose	positive
	Maltose	positive
55	Lactose	positive
	Melibiose	negative

(continued)

[Oxidation characteristics]	
5	Sucrose negative
	Trehalose positive
	Inulin negative
	Melezitose negative
10	D-Raffinose negative
	Amidon negative
	Glycogen negative
	Xylitol negative
	$\beta$ -Gentiobiose positive
15	D-Turanose negative
	D-Lyxose negative
	D-Tagatose negative
	D-Fucose positive
20	L-Fucose negative
	D-Arabitol positive
	L-Arabitol positive
	Gluconic acid negative
25	2-Ketogluconic acid negative
	5-Ketogluconic acid negative

[0082] The taxonomical position of the KS1 strain having the aforementioned bacteriological characteristics was investigated with reference to the Bergey's Manual of Determinative Bacteriology, and the strain was identified to belong to the genus *Burkholderia*, and was a bacterial strain of *Burkholderia cepacia*.

[0083] The genus *Burkholderia* was conventionally classified into the genus *Pseudomonas*, but is separately classified as the genus *Burkholderia* at present (Yabuuchi, E., Kosako, Y., Oyaizu, H., Yano, I., Hotta, H., Hashimoto, Y., Ezaki, T. and Arakawa, M., Microbiol. Immunol. Vol. 36 (12): 1251-1275 (1992); International Journal of Systematic Bacteriology, Apr., 1993, pp.398-399).

[0084] Further, the inventors of the present invention obtained several *Burkholderia cepacia* strains other than the *Burkholderia cepacia* KS1 strain, which were deposited at the Institute for Fermentation, Osaka or the Japan Collection of Microorganisms (JCM), Institute of Physical and Chemical Research, and measured glucose dehydrogenase activities of the strains, and they were confirmed to have the activity. The glucose dehydrogenase activity was measured by the method described later in Example 2. Relative activities of these strains based on the enzymatic activity of a water-soluble fraction of the KS1 strain, which is taken as 100, are shown in Table 1.

40

Table 1

Bacterial strain		Glucose dehydrogenase activity	
		70° C	45°C
45	KS1 Water-soluble fraction	100	100
	JCM5506 Water-soluble fraction	100	100
		100	100
50	JCM5507 Water-soluble fraction	100	100
		100	100
	JCM2800 Water-soluble fraction	100	100
	JCM2801 Water-soluble fraction	100	100
55	IFO15124 Water-soluble fraction	100	100
	IFO14595 Water-soluble fraction	100	100

Example 2: Extraction of glucose dehydrogenase

## &lt;1&gt; Culture of cells

- 5 [0085] As the culture conditions of the bacterium, usual aerobic culture conditions were used. The cells were cultured at 34°C for 8 hours in 7 L of a medium containing the following ingredients per liter.

10	Polypeptone	10 g
	Yeast extract	1 g
	NaCl	5 g
	KH <sub>2</sub> PO <sub>4</sub>	2 g
	Glucose	5 g
15	Einol (ABLE Co., Tokyo, Japan)	0.14 g
	Total volume including distilled water	1 L
	Adjusted pH	7.2

- 20 [0086] In a volume of 7 L of the culture broth was centrifuged at 9,000 x g at 4°C for 10 minutes to obtain about 60 g of cells.

## &lt;2&gt; Preparation of roughly purified fraction

- 25 [0087] In an amount of 60 g of the cells were dispersed in 10 mM potassium phosphate buffer (pH 6.0), and a pressure difference of 1,500 Kg/cm<sup>2</sup> was applied to the cells by using a French press (Otake Corporation, Tokyo, Japan) to disrupt cell membranes. The cell extract was centrifuged at 8000 x g for 10 minutes to remove cellular solid. Further, the supernatant was subjected to ultracentrifugation at 69,800 x g at 4°C for 90 minutes to obtain about 8 g of a membrane fraction as precipitates.

## &lt;3&gt; Purification of enzyme

- 30 [0088] The membrane fraction was redispersed in 10 mM potassium phosphate buffer (pH 6.0) containing 1% of Triton X-100 as a final concentration. Then, the dispersion was slowly stirred overnight at 4°C. After the dispersion was subjected to ultracentrifugation (69,800 x g, 4°C, 90 minutes), the solubilized membrane fraction was centrifuged again at 4°C for 15 minutes at 15,000 x g to obtain a supernatant.

- 35 [0089] The solubilized membrane fraction was added with the same volume of 10 mM potassium phosphate buffer (pH 8.0) containing 0.2% Triton X-100. The solution was dialyzed, and then applied to a DEAE-TOYOPEARL column (22 mm ID x 20 cm, Tosoh Corporation, Tokyo, Japan) equalized with 10 mM potassium phosphate buffer (pH 8.0) containing 0.2% Triton X-100. Proteins were eluted with a linear gradient of 0 to 0.15 M NaCl in 10 mM potassium phosphate buffer (pH 8.0). The flow rate was 5 ml/min. GDH was eluted at a NaCl concentration of about 75 mM.

- 40 Fractions exhibiting the GDH activity were collected and dialyzed overnight against 10 mM potassium phosphate buffer (pH 8.0, 4°C) containing 0.2% Triton X-100.

- [0090] Further, the dialyzed enzyme solution was applied to a DEAE-5PW column (8.0 mm ID x 7.5 cm, Tosoh Corporation, Tokyo, Japan). This column was equilibrated beforehand with 10 mM potassium phosphate buffer (pH 6.0) containing 0.2% Triton X-100. The proteins were eluted with a linear gradient of 0 to 100 mM NaCl in 10 mM potassium phosphate buffer (pH 8.0). The flow rate was 1 ml/min. Fractions exhibiting the GDH activity were eluted at a NaCl concentration of about 20 mM. The fractions having the GDH activity were collected and desalting overnight with 10 mM potassium phosphate buffer (pH 8.0) containing 0.2% Triton X-100 to obtain the purified enzyme.

- [0091] The GDH activity was measured according to the following method throughout this example and the following examples.

- 50 [0092] As electron acceptors, 2,6-dichlorophenol-indophenol (DCIP) and phenazine methosulfate (PMS) were used. The reaction was allowed in a polyethylene tube at a predetermined temperature. In a volume of 5 µl of the enzyme solution was added to 20 µl of 25 mM Tris-HCl buffer (pH 8.0) containing 0.75 mM PMS and 0.75 mM DCIP. This mixture was left for 1 minute beforehand at a constant temperature. The reaction was started with the addition of 1 µl of 2 M glucose (final concentration: 77 mM) and left at a constant temperature for 2 minutes. Subsequently, 100 µl of ice-cooled distilled water or 120 µl of 7.5 M urea was added to cool the sample. A reduction reaction of the electron acceptors due to the dehydrogenation of glucose was monitored by using an ultra-micro measurement cell (100 µl) and a spectrophotometer (UV160, Shimadzu Corporation, Kyoto, Japan) that enabled measurement using the cell. That is, decoloration with time due to the reduction of DCIP was measured at 600 nm, which is the absorption wave-

length of DCIP. The molar absorbance coefficient of DCIP ( $22.23 \text{ mM} \times \text{cm}^{-1}$ ) was used. One unit (U) of the enzyme was defined as the amount of oxidizing  $1 \mu\text{M}$  of glucose per minute under standard test conditions. The protein concentration was measured by the Lowry method.

5    Example 3

[0093] Native PAGE electrophoresis was performed for the purified enzyme. The electrophoresis was performed on 8 to 25% polyacrylamide gradient gel using a Tris-alanine buffer system containing 1% Triton X-100. The gel was stained with silver nitrate. As protein markers, thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa) and chymotrypsinogen A (25 kDa) were used.

[0094] Further, activity staining was performed for the native PAGE gel by incubating the gel in the following solution for 30 minutes. At GDH activity sites, nitroblue tetrazolium was reduced and formazan was produced, resulting in development of dark purple color.

15	<table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="padding: 2px;">200 mM</td><td style="padding: 2px;">glucose</td></tr> <tr> <td style="padding: 2px;">0.1 mM</td><td style="padding: 2px;">nitroblue tetrazolium</td></tr> <tr> <td style="padding: 2px;">0.3 mM</td><td style="padding: 2px;">phenazine methosulfate</td></tr> <tr> <td style="padding: 2px;">20 mM</td><td style="padding: 2px;">Tris-HCl buffer (pH 8.0)</td></tr> </table>	200 mM	glucose	0.1 mM	nitroblue tetrazolium	0.3 mM	phenazine methosulfate	20 mM	Tris-HCl buffer (pH 8.0)
200 mM	glucose								
0.1 mM	nitroblue tetrazolium								
0.3 mM	phenazine methosulfate								
20 mM	Tris-HCl buffer (pH 8.0)								

20 [0095] From the results of the silver staining in the native PAGE, it was estimated that the enzyme consisted of a single kind of enzyme and had a molecular weight of about 400 kDa. Further, when the gel was stained for the activity, the activity was observed at a site of the same mobility as in the silver staining (See Fig. 1. In the figure, Lane 1 shows the results of silver staining of marker proteins having standard molecular weights, Lane 2 shows the silver staining of the enzyme, and Lane 4 shows the staining for activity of the enzyme). When the enzyme was heated at  $70^\circ\text{C}$  for 30 minutes, the activity unexpectedly remained, and the enzyme was separated into proteins one of which had the activity and showed a molecular weight of about 85 kDa (See Fig. 1. In the figure, Lane 3 shows the results of the silver staining of the enzyme heated at  $70^\circ\text{C}$  for 30 minutes, and Lane 5 shows the staining for activity of the enzyme heated at  $70^\circ\text{C}$  for 30 minutes). These results suggest that the enzyme consists of subunits.

25    Example 4

[0096] The purified enzyme solution was subjected to SDS-PAGE. SDS-PAGE was performed in 8 to 25% gradient polyacrylamide gel by using a Tris-tricine buffer. Proteins in the gel were stained with silver nitrate. Separation and development were automatically performed by using Phast System (Pharmacia). The molecular mass was determined based on the relative migrations of the standard proteins. The enzyme was separated into proteins having molecular weights of about 60 kDa and 43 kDa by SDS-PAGE (See Fig. 2. Fig. 2 is an electrophoretic photograph. In the figure, Lane 1 shows the results of the silver nitrate staining of the marker proteins having standard molecular weights, and Lane 2 shows the results of the silver nitrate staining of the enzyme). Thus, it was suggested that the  $\alpha$ -subunit of 60 kDa and the  $\beta$ -subunit of 43 kDa were bound in the enzyme, and it was expected that an octamer was formed by four each of these subunits bonding to each other.

[0097] The  $\beta$ -subunit, a protein of 43 kDa separated by SDS-PAGE, was transferred onto a polyvinylidene fluoride membrane, and then the amino acid sequence at the N-terminus of the  $\beta$ -subunit was determined by using an amino acid sequencer (PPSQ-10, Shimadzu Corporation). As a result, it was found that the amino acid sequence at the N-terminus of the protein consisted of 16 residues of the amino acid sequence of SEQ ID NO: 5.

[0098] Further, the results obtained with the enzyme subjected to a heat treatment at  $70^\circ\text{C}$  for 30 minutes are shown as Lane 3 in Fig. 2. Based on this result of SDS-PAGE, it can be estimated that the enzyme was changed into a single polypeptide having a molecular weight of 60 kDa after the heat treatment.

30    Example 5

[0099] The enzyme was subjected to gel filtration chromatography. As the gel, TSK Gel G3000SW (Tosoh Corporation) was used, and the gel column (8.0 mm ID x 30 cm Tosoh Corporation, Tokyo, Japan) was equilibrated with a solution containing 0.3 M NaCl and 0.1% Triton X-100 in 10 mM potassium phosphate buffer (pH 6.0). Fractions (125  $\mu\text{l}$ ) were collected. Seven kinds of protein markers were used to determine the molecular weight of the purified enzyme. As the protein markers, thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa) and chymotrypsinogen A (25 kDa) were used.

[0100] It was confirmed that the molecular weight of the enzyme was about 380 kDa.

Example 6

[0101] The optimal temperature of the purified enzyme was examined.

5 [0102] The enzyme was incubated beforehand in Tris-HCl buffer (pH 8.0) at a predetermined temperature for 1 minute, and then the reaction was started. The activity was measured at a predetermined reaction temperature. The optimal temperature was observed around 45°C (see Fig. 3 (a)). Further, a peak was also observed around 75°C, although the activity was lower than the activity around 45°C.

10 [0103] Further, in order to examine thermal stability of the enzyme, the enzyme was left at each constant temperature for 30 minutes, and the residual enzymatic activity was measured at 45°C (see Fig. 3 (b)).

Example 7

15 [0104] The optimal temperature and the thermal stability of the peptide enzyme constituting the single oligopeptide having a molecular weight of 60 kDa obtained by heating the enzyme at 70°C for 30 minutes were examined.

[0105] This peptide enzyme showed an optimal temperature higher than that of the unheated enzyme as well as thermal stability. There has been no report about an enzyme having such temperature dependency.

20 [0106] The enzyme was incubated beforehand in Tris-HCl buffer (pH 8.0) at a predetermined temperature for 1 minute, and then the reaction was started. The activity was measured at a predetermined reaction temperature. The optimal temperature was observed around 75°C (see Fig. 4 (a)).

[0107] Further, in order to examine thermal stability of the enzyme, the enzyme was left at each constant temperature for 30 minutes, and the residual enzymatic activity was measured at 70°C (see Fig. 4 (b)).

Example 8

25 [0108] In order to investigate a role of each subunit, spectrophotometric analysis was performed for GDH before and after the heat treatment. Figs. 5 (a) and (b) show absorptions of oxidized and reduced GDHs before and after heat treatment (in the presence of glucose). The absorption wavelength of the oxidized GDH before heat treatment, which was the original GDH, showed a characteristic peak at 409 nm. Further, the peak shifted to 417 nm in the presence of glucose, and two more peaks were observed at 523 nm and 550 nm (Fig. 5 (a)). In contrast, the GDH after the heat treatment no longer showed the characteristic peak at 409 nm (Fig. 5 (b)), and no significant difference was observed between the oxidized and reduced GDHs.

[0109] The absorption wavelength of the oxidized GDH before heat treatment, which was the original GDH, was similar to the absorption wavelength of alcohol dehydrogenase or aldehyde dehydrogenase comprising a dehydrogenase cytochrome complex of *Gluconobacter sp.* or *Acetobacter sp.* (refer to the following references: Adachi, O., Tayama, K., Shinagawa, E., Matsushita, K. and Ameyama, M., Agr. Biol. Chem., 42, 2045-2056 (1978); Adachi, O., Miyagawa, E., Matsushita, K. and Ameyama, M., Agr. Biol. Chem., 42, 2331-2340 (1978); Ameyama, M. and Adachi, O., Methods Enzymol., 89, 450-457 (1982); Adachi, O., Tayama, K., Shinagawa, E., Matsushita, K. and Ameyama, M., Agr. Biol. Chem., 44, 503-515 (1980); Ameyama, M. and Adachi, O., Methods Enzymol., 89, 491-497 (1982)).

35 [0110] The results indicated a possibility that the oligomer complex of the GDH contained cytochrome. Therefore, it can be considered that the observed wavelength similar to that of cytochrome C is attributable to the β-subunit and was lost during the heat treatment, and thus the β-subunit consists of cytochrome C.

Example 9

45 [0111] A band containing the β-subunit obtained by the electrophoresis in Example 4 was excised, and the amino acid sequence was analyzed by using a peptide sequencer (PPSQ-10, Shimadzu Corporation). As a result, the N-terminus amino acid sequence consisting of 16 residues shown in SEQ ID NO: 5 could be obtained.

[0112] It was attempted to amplify a gene region encoding the aforementioned N-terminus amino acid sequence of 16 residues by PCR based on the peptide sequence. That is, two of PCR primers were designed, which had a nucleotide sequence on the forward side (SEQ ID NO: 6) corresponding to 5 residues at the N-terminus and a nucleotide sequence on the reverse side (SEQ ID NO: 7) corresponding to the antisense strand of 5 residues at the C-terminus in the peptide chain of the 16 residues. When PCR was performed in a conventional manner for the KS1 strain genome by using this pair of PCR primers, a gene fragment of about 50 bp was amplified. When the nucleotide sequence of this gene fragment was determined in a conventional manner, a nucleotide sequence of 58 nucleotides containing the pair of PCR primers were decoded. Among these nucleotides, 18 nucleotides excluding the PCR primers were analyzed, and a gene sequence corresponding to a region from Pro, which was the 6th residue from the N-terminus side of the aforementioned 16 residues at the N-terminus of the β-subunit, to Arg, which was the 11th residue, was found (SEQ ID NO: 8). Thus, it was found that the amplified gene fragment included the gene fragment of the β-subunit.

[0113] Further, it was also found that the  $\beta$ -subunit existed after 22 amino acid residues following the  $\alpha$ -subunit. This was based on a finding that, since the amino acid sequence at the N-terminus of the purified  $\beta$ -subunit determined in Example 4 matched 5 amino acid residues translated from the nucleotide sequence of the nucleotide numbers 2452 to 2466 in SEQ ID NO: 1, these sequences are identical.

5 [0114] Furthermore, it is inferred that the nucleotide sequence of the nucleotide numbers 2386 to 2451 in SEQ ID NO: 1 is the signal peptide of the  $\beta$ -subunit. The amino acid sequence encoded by this nucleotide sequence corresponds to the amino acid numbers 1 to 22 in the amino acid sequence of SEQ ID NO: 4.

Example 10

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[0115] The purified enzyme and a commercially available NAD coenzyme GDH (abbreviated as "NAD-GDH") were added and mixed in 50 mM potassium phosphate buffer (pH 7.5) containing 0.1% Triton X-100 and 1 mM  $\text{CaCl}_2$  at a concentration of 100 U/L each. Each solution was placed in a hot tank at 60°C, and the residual activity was measured.

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Table 2:

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Residual relative activity (%)		
Time (min)	NAD-GDH	The enzyme GDH
0	100	100
15	20	100
30	5	100

25 [0116] It was confirmed that the enzyme had surprising thermal stability in comparison with that of the currently commercially available GDH enzyme. It was found that the enzyme was a novel enzyme that is totally different from the commercially available NAD-GDH.

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Example 10: Isolation of gene encoding  $\alpha$ -subunit of GDH

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<1> Preparation of chromosomal DNA from *Burkholderia cepacia* KS1 strain

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[0117] A chromosomal gene was prepared from the *Burkholderia cepacia* KS1 strain in a conventional manner. That is, the bacterial strain was shaken overnight at 34°C by using a TL liquid medium (10 g of polypeptone, 1 g of yeast extract, 5 g of NaCl, 2 g of  $\text{KH}_2\text{PO}_4$ , 5 g of glucose in 1 L, pH 7.2). The grown cells were collected by using a centrifugal machine. The cells were suspended in a solution containing 10 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5% SDS and 100  $\mu\text{g}/\text{ml}$  proteinase K and treated at 50°C for 6 hours. This mixture was added with an equivalent volume of phenolchloroform and stirred at room temperature for 10 minutes, and then the supernatant was collected by using a centrifugal machine. The supernatant was added with sodium acetate at a final concentration of 0.3 M and overlaid with two-fold volume of ethanol to precipitate chromosomal DNA in the intermediate layer. The DNA was taken up with a glass rod, washed with 70% ethanol and dissolved in an appropriate amount of TE buffer to obtain a chromosomal DNA solution.

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<2> Determination of N-terminus amino acid sequence of  $\alpha$ -subunit of GDH

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[0118] GDH purified in the same manner as in Example 2 was concentrated by lyophilization and developed by SDS-electrophoresis using 12.5% polyacrylamide to isolate the  $\alpha$ -subunit. The  $\alpha$ -subunit thus obtained was transferred onto a polyvinylidene fluoride membrane, and then the N-terminus amino acid sequence was determined by using an amino acid sequencer (PPSQ-10, Shimadzu Corporation). As a result, it was found that the enzyme included a peptide sequence consisting of 11 residues of the amino acid numbers 2 to 12 in the amino acid sequence of SEQ ID NO: 3.

<3> Cloning of gene encoding  $\alpha$ -subunit

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[0119] In an amount of 1  $\mu\text{g}$  of the DNA prepared in <1> was subjected to limited digestion with a restriction enzyme Sau3AI and treated with calf intestinal alkaline phosphatase (CIAP). Separately, SuperCosI (obtained from STRATA-GENE), which is a cosmid, was treated with BamHI, and the DNA fragment obtained by the limited digestion of the chromosomal DNA fragment derived from the  $\alpha$ -15 strain with Sau3AI was incorporated into SuperCosI by using T4 DNA ligase. *Escherichia coli* XL-1 Blue MR (obtained from STRATAGENE) was transformed with the obtained recom-

binant DNA. Transformants were selected on an LB agar medium containing 10 µg/ml neomycin and 25 µg/ml ampicillin based on neomycin resistance and ampicillin resistance, which are antibiotic resistances of SuperCosI. The obtained transformants were cultured in the LB liquid medium. These transformant cells were collected and suspended in a reagent for measuring the GDH activity, and clones were selected by using dehydrogenase activity for glucose as an index. As a result, one clone strain showing the glucose dehydrogenase activity was obtained.

**<4> Subcloning**

[0120] DNA fragments containing the target gene were prepared from the cosmid, SuperCosI, containing the gene encoding the  $\alpha$ -subunit obtained in <3>. The inserted gene fragments were excised from the cosmid by using a restriction enzyme NotI. These DNA fragments were treated with a restriction enzyme XbaI and incorporated into plasmid pUC18 digested with XbaI. The *Escherichia coli* DH5 $\alpha$ MCR strain was transformed with the plasmid pUC18 containing each insert fragment, and colonies grown on an LB agar medium containing 50 µg/ml ampicillin were collected. The obtained transformants were cultured in a liquid LB medium and examined for the GDH activity in the cells in the same manner as in <3>. As a result, a strain showing the GDH activity was obtained from one transformant. The plasmid was extracted from this transformant, and the inserted DNA fragment was analyzed. As a result, an insert fragment of about 8.8 kbp was confirmed. This plasmid was designated as pKS1.

**<5> Determination of nucleotide sequence**

[0121] The nucleotide sequence of the inserted DNA fragment in pKS1 was determined according to the restriction enzyme analysis and a conventional method. As a result, the sequence of the DNA encoding the N-terminus amino acid sequence of the  $\alpha$ -subunit found in <2> was confirmed in this inserted DNA fragment, and an open reading frame containing this sequence was found. The determined nucleotide sequence and the amino acid sequence that can be encoded by this nucleotide sequence are as shown in SEQ ID NOS: 1 and 3. The molecular weight of a protein obtained from the amino acid sequence was 59,831 Da and substantially matched the molecular weight of 60 kDa obtained by SDS-PAGE of the  $\alpha$ -subunit of the *Burkholderia cepacia* KS1 strain.  
[0122] Since the nucleotide sequence of the  $\alpha$ -subunit was determined, a vector was produced by using the aforementioned structural gene of the  $\alpha$ -subunit, and a transformant was further produced with this vector.  
[0123] First, a gene to be inserted into the vector was prepared as follows.  
[0124] Amplification was performed by PCR using a genome fragment derived from the KS1 strain as a template so that a desired restriction enzyme site should be included. The following pair of oligonucleotide primers were used in PCR.

35

(Forward)

5' -CCCAAGCTTGGGCCGATACCGATAACGCA-3' (SEQ ID NO: 9)

40

(Reverse)

5' -GAGAAGCTTCCGCACGGTCAGACTTCC-3' (SEQ ID NO: 10)

45

[0125] The gene amplified by PCR was digested with a restriction enzyme HindIII and inserted into the expression vector pFLAG-CTS (SIGMA) at its cloning site, HindIII site. The obtained plasmid was designated as pFLAG-CTS/ $\alpha$ .  
[0126] The *Escherichia coli* DH5 $\alpha$ MCR strain was transformed with the aforementioned plasmid pFLAG-CTS/ $\alpha$ , and a colony grown on an LB agar medium containing 50 µg/ml ampicillin was collected.  
[0127] Further, when the open reading frame of the pKS1 insert fragment was searched in the upstream of the  $\alpha$ -subunit, a structural gene of 507 nucleotides encoding a polypeptide comprising 168 amino acid residues shown in SEQ ID NO: 2 (nucleotide numbers 258 to 761 in SEQ ID NO: 1) was newly found. This structural gene was considered to encode the  $\gamma$ -subunit.  
[0128] Since it was found that the region encoding the  $\gamma$ -subunit existed upstream from the coding region of the  $\alpha$ -subunit, a recombinant vector containing a gene having a polycistronic structure continuously including the  $\gamma$ -subunit and the  $\alpha$ -subunit was produced, and a transformant introduced with this vector was constructed.  
[0129] First, a gene to be inserted into the vector was prepared as follows.

[0130] Amplification was performed by PCR using a genome fragment of the KS1 strain continuously including the structural gene of the  $\gamma$ -subunit and the structural gene of the  $\alpha$ -subunit as a template so that a desired restriction enzyme site should be included. The following pair of oligonucleotide primers were used for PCR.

5

(Forward)

5'-CATGCCATGGCACACAACGACAACACT-3' (SEQ ID NO: 11)

10

(Reverse)

5'-CCCAAGCTTGGGTCAAGACTTCCTTCTTCAGC-3' (SEQ ID NO: 12)

15

- [0131] The 5' end and the 3' end of the gene amplified by PCR were digested with Ncol and HindIII, respectively, and the gene was inserted into the vector pTrc99A (Pharmacia) at its cloning site, Ncol/HindIII site. The obtained plasmid was designated as pTrc99A/ $\gamma+\alpha$ .  
 20 [0132] The *Escherichia coli* DH5 $\alpha$ MCR strain was transformed with the aforementioned plasmid pTrc99A/ $\gamma+\alpha$ , and a colony grown on an LB agar medium containing 50  $\mu$ g/ml ampicillin was collected.

Example 11: Production of  $\alpha$ -subunit of GDH by recombinant *Escherichia coli*

- 25 [0133] The  $\alpha$ -subunit was produced by using the *Escherichia coli* DH5 $\alpha$ MCR strain transformed with each of the aforementioned plasmids pKS1, pFLAG-CTS/ $\alpha$  and pTrc99A/ $\gamma+\alpha$ . Each transformant was inoculated into 3 ml of LB medium containing 50  $\mu$ g/ml ampicillin and cultured at 37°C for 12 hours, and cells were collected by using a centrifugal machine. The cells were disrupted by using a French press (1500 kgf), and a membrane fraction (10 mM potassium phosphate buffer, pH 6.0) was isolated by ultracentrifugation (160,400 x g, 4°C, 90 minutes).

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Example 12: Assay of glucose

- 35 [0134] First, the GDH activity in each of the aforementioned membrane fractions was confirmed. Specifically, visual determination was performed by using a 10 mM potassium phosphate buffer (pH 7.0) containing 594  $\mu$ M methylphenazone methosulfate (mPMS) and 5.94  $\mu$ M 2,6-dichlorophenol-indopheol (DCIP). The results are shown below. The number of + represents the degree of color change from blue to colorless.

40	Membrane fraction of cultured transformant transformed with pFLAG-CTS/ $\alpha$ Membrane fraction of cultured transformant transformed with pKS1 Membrane fraction of cultured transformant transformed with pTrc99A/ $\gamma+\alpha$	+ ++ +++
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- 45 [0135] The GDH activity of the membrane fraction of the cultured transformant transformed with pFLAG-CTS/ $\alpha$  incorporated only with the  $\alpha$ -subunit was the lowest, and the GDH activity of the membrane fraction of the cultured transformant transformed with pTrc99A/ $\gamma+\alpha$ , with which a vector was efficiently constructed, was the highest.

- [0136] Although the  $\alpha$ -subunit was expressed even in the transformant transformed with a vector using only the structural gene of the  $\alpha$ -subunit, the  $\alpha$ -subunit could be efficiently obtained by using a vector containing the structural gene of the  $\gamma$ -subunit and the structural gene of the  $\alpha$ -subunit in combination.

- 50 [0137] Glucose was assayed by using the glucose dehydrogenase of the present invention. The enzymatic activity of the glucose dehydrogenase ( $\alpha$ -subunit) of the present invention was measured by using glucose at various concentrations. The GDH activity was measured in 10 mM potassium phosphate buffer (pH 7.0) containing 594  $\mu$ M methylphenazone methosulfate (mPMS) and 5.94  $\mu$ M 2,6-dichlorophenol-indopheol (DCIP). An enzyme sample and glucose as a substrate were added and incubated at 37°C, and change in the absorbance of DCIP at 600 nm was monitored by using a spectrophotometer. The absorbance decreasing rate was measured as an enzymatic reaction rate. Glucose could be quantified in the range of 0.01 to 1.0 mM by using the GDH of the present invention.

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Example 13: Preparation and evaluation of glucose sensor

- [0138] The glucose dehydrogenase (25 units) of the present invention obtained in Example 2 was added with 20 mg of carbon paste and lyophilized. These were sufficiently mixed, applied only on a surface of a carbon paste electrode already filled with about 40 mg of carbon paste and polished on a filter paper. This electrode was treated in 10 mM MOPS buffer (pH 7.0) containing 1% glutaraldehyde at room temperature for 30 minutes and then treated in 10 mM MOPS buffer (pH 7.0) containing 20 mM lysine at room temperature for 20 minutes to block glutaraldehyde. This electrode was equilibrated in 10 mM MOPS buffer (pH 7.0) at room temperature for 1 hour or longer. The electrode was stored at 4°C.
- [0139] By using the aforementioned electrode as a working electrode, an Ag/AgCl electrode as a reference electrode and a Pt electrode as a counter electrode, a response current value was measured upon addition of glucose. The 10 mM potassium phosphate buffer containing 1 mM methoxy-PMS was used as the reaction solution, and a potential of 100 mV was applied for the measurement.
- [0140] Glucose concentration was measured by using the produced enzyme sensor. Glucose could be quantified in the range of 0.05 to 5.0 mM by using the enzyme sensor on which the glucose dehydrogenase of the present invention was immobilized (Fig. 6).

Example 14: Preparation and evaluation of glucose sensor by GDH obtained from transformant

- [0141] In an amount of 10 U of the  $\alpha$ -subunit (249 U/mg protein) of the present invention obtained in Example 12 was added with 50 mg of carbon paste and lyophilized. These were sufficiently mixed, applied only on a surface of a carbon paste electrode already filled with about 40 mg of carbon paste and polished on a filter paper. This electrode was treated in a 10 mM MOPS buffer (pH 7.0) containing 1% glutaraldehyde at room temperature for 30 minutes and then treated in 10 mM MOPS buffer (pH 7.0) containing 20 mM lysine at room temperature for 20 minutes to block glutaraldehyde. This electrode was equilibrated in 10 mM MOPS buffer (pH 7.0) at room temperature for 1 hour or longer. The electrode was stored at 4°C.
- [0142] By using the aforementioned electrode as a working electrode, an Ag/AgCl electrode as a reference electrode and a Pt electrode as a counter electrode, a response current value was measured upon addition of glucose. The 10 mM potassium phosphate buffer containing 1 mM methoxy-PMS was used as the reaction solution, and the measurement was performed for glucose aqueous solutions of various concentrations at 25°C and 40°C with applying a potential of 100 mV.
- [0143] It was confirmed that, when the glucose concentration was measured by using the produced enzyme sensor, a current corresponding to each concentration was obtained.

35 Industrial Applicability

- [0144] According to the present invention, an enzyme that has high substrate specificity, can be produced at a low cost and is not affected by oxygen dissolved in a measurement sample, in particular, novel glucose dehydrogenase having superior thermal stability, and a method for producing the enzyme could be provided. Further, a novel bacterial strain of *Burkholderia cepacia* producing the enzyme was obtained. A glucose sensor effective for measurement of glucose can also be provided by using an enzyme electrode containing the enzyme or the bacterial strain.
- [0145] Further, since the glucose dehydrogenase gene, a peptide that enables efficient expression of the gene and a DNA encoding this peptide were found by the present invention, a large amount of GDH can be prepared by using recombinant DNA techniques based on the gene.

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 tacatttcag gtcgcgcgg attttttaga aataatacgc ggggtttcc cgaatccgg 240  
 gtgcgagaga aggaaac atg cac aac gac aac act ccc cac tgc cgt cgc 290  
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 His Gly Asp Ala Ala Ala Ser Gly Ile Thr Arg Arg Gln Trp Leu Gln  
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 Pro Asp Lys Arg Ile Val Ala Ala Leu Tyr Lys Asp Lys Thr Gly Ala  
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 Glu Thr Pro Lys Ile Leu Leu Met Ser Ala Asn Arg Asp Phe Pro Asn  
 55

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	325	330	335
	Arg Gly Pro Gln Glu Met Thr Ser Leu Ile Gly Phe Arg Asp Gly Pro		
10	340	345	350
	Phe Arg Ala Thr Glu Ala Ala Lys Lys Ile His Leu Ser Asn Leu Ser		
	355	360	365
	Arg Ile Asp Gln Glu Thr Gln Lys Ile Phe Lys Ala Gly Lys Leu Met		
15	370	375	380
	Lys Pro Asp Glu Leu Asp Ala Gln Ile Arg Asp Arg Ser Ala Arg Tyr		
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	Val Gln Phe Asp Cys Phe His Glu Ile Leu Pro Gln Pro Glu Asn Arg		
20	405	410	415
	Ile Val Pro Ser Lys Thr Ala Thr Asp Ala Ile Gly Ile Pro Arg Pro		
	420	425	430
	Glu Ile Thr Tyr Ala Ile Asp Asp Tyr Val Lys Arg Gly Ala Ala His		
25	435	440	445
	Thr Arg Glu Val Tyr Ala Thr Ala Ala Lys Val Leu Gly Gly Thr Asp		
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	480		
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## Claims

1. A method for producing glucose dehydrogenase comprising the steps of culturing a microorganism belonging to the genus *Burkholderia* and having glucose dehydrogenase producing ability in a medium, and collecting glucose dehydrogenase from the medium and/or cells of the microorganism.
- 5 2. The method for producing glucose dehydrogenase according to claim 1, wherein the microorganism is *Burkholderia cepacia*.
- 10 3. The method for producing glucose dehydrogenase according to claim 1 or 2, wherein the glucose dehydrogenase has the following properties:
  - (i) the enzyme has an action of catalyzing dehydrogenation reaction of glucose;
  - (ii) the enzyme consists of subunits showing a molecular weight of about 60 kDa and a molecular weight of about 43 kDa in SDS-polyacrylamide gel electrophoresis under a reducing condition;
  - 15 (iii) the enzyme shows a molecular weight of about 380 kDa in gel filtration chromatography using TSK Gel G3000SW (Tosoh Corporation); and
  - (iv) the enzyme shows an optimal reaction temperature around 45°C (Tris-HCl buffer, pH 8.0).
- 20 4. The method for producing glucose dehydrogenase according to claim 3, wherein the subunit showing a molecular weight of about 43 kDa is an electron-transferring protein.
5. The method for producing glucose dehydrogenase according to claim 4, wherein the electron-transferring protein is cytochrome C.
- 25 6. A glucose dehydrogenase, which can be produced by a microorganism belonging to the genus *Burkholderia*.
7. The glucose dehydrogenase according to claim 6, wherein the microorganism is *Burkholderia cepacia*.
- 30 8. The glucose dehydrogenase according to claim 6 or 7, wherein the glucose dehydrogenase has the following properties:
  - (i) the enzyme has an action of catalyzing dehydrogenation reaction of glucose;
  - (ii) the enzyme consists of subunits showing a molecular weight of about 60 kDa and a molecular weight of about 43 kDa in SDS-polyacrylamide gel electrophoresis under a reducing condition;
  - 35 (iii) the enzyme shows a molecular weight of about 380 kDa in gel filtration chromatography using TSK Gel G3000SW (Tosoh Corporation); and
  - (iv) the enzyme shows an optimal reaction temperature around 45°C (Tris-HCl buffer, pH 8.0).
- 40 9. The glucose dehydrogenase according to claim 8, wherein the subunit showing a molecular weight of about 43 kDa is an electron-transferring protein.
10. The glucose dehydrogenase according to claim 9, wherein the electron-transferring protein is cytochrome C.
- 45 11. The glucose dehydrogenase according to any one of claims 8 to 10, wherein the subunit showing a molecular weight of about 60 kDa comprises the amino acid sequence of the amino acid numbers 2 to 12 in SEQ ID NO: 3.
12. The glucose dehydrogenase according to any one of claims 8 to 11, wherein the N-terminus of the subunit showing a molecular weight of 43 kDa has the amino acid sequence of SEQ ID NO: 5.
- 50 13. The glucose dehydrogenase according to claim 11, wherein the subunit showing a molecular weight of about 60 kDa is a protein defined in the following (A) or (B):
  - (A) a protein which has the amino acid sequence of SEQ ID NO: 3;
  - (B) a protein which has the amino acid sequence of SEQ ID NO: 3 including substitution, deletion, insertion or addition of one or several amino acid residues and a glucose dehydrogenase activity.
14. The glucose dehydrogenase according to claim 6, which shows activity peaks around 45°C and around 75°C.

15. A cytochrome C, which is a subunit of the glucose dehydrogenase according to claim 10 and has the amino acid sequence of SEQ ID NO: 5.
- 5      16. A DNA encoding a part of the cytochrome C according to claim 15 and having the nucleotide sequence of SEQ ID NO: 8.
17. A DNA encoding a part of the cytochrome C according to claim 15 and having the nucleotide sequence of the nucleotide numbers 2386 to 2467 in the nucleotide sequence of SEQ ID NO: 1.
- 10     18. A DNA encoding a signal peptide of the cytochrome C according to claim 15 and comprising the nucleotide sequence of the nucleotide numbers 2386 to 2451 in the nucleotide sequence of SEQ ID NO: 1.
19. A peptide which is a signal peptide of cytochrome C and has the amino acid sequence of the amino acid numbers 1 to 22 in the amino acid sequence of SEQ ID NO: 4.
- 15     20. A protein having the following properties:
- (i) the protein can constitute the glucose dehydrogenase according to claim 6 as a subunit;
- (ii) the protein has a glucose dehydrogenase activity;
- 20     (iii) the protein shows a molecular weight of about 60 kDa in SDS-polyacrylamide gel electrophoresis under a reducing condition; and
- (iv) the protein shows an optimal reaction temperature around 75°C (Tris-HCl buffer, pH 8.0).
21. The protein according to claim 20, which comprises the amino acid sequence of the amino acid numbers 2 to 12 in SEQ ID NO: 3.
- 25     22. The glucose dehydrogenase according to claim 21, wherein the protein is a protein defined in the following (A) or (B):
- 30       (A) a protein which has the amino acid sequence of SEQ ID NO: 3;
- (B) a protein which has the amino acid sequence of SEQ ID NO: 3 including substitution, deletion, insertion or addition of one or several amino acid residues and a glucose dehydrogenase activity.
23. A protein defined in the following (A) or (B):
- 35       (A) a protein which has the amino acid sequence of SEQ ID NO: 3;
- (B) a protein which has the amino acid sequence of SEQ ID NO: 3 including substitution, deletion, insertion or addition of one or several amino acid residues and a glucose dehydrogenase activity.
- 40     24. A DNA encoding a protein defined in the following (A) or (B):
- (A) a protein which has the amino acid sequence of SEQ ID NO: 3;
- (B) a protein which has the amino acid sequence of SEQ ID NO: 3 including substitution, deletion, insertion or addition of one or several amino acid residues and a glucose dehydrogenase activity.
- 45     25. The DNA according to claim 24, which is a DNA defined in the following (a) or (b):
- (a) a DNA which comprises the nucleotide sequence of the nucleotide numbers 764 to 2380 in the nucleotide sequence of SEQ ID NO: 1;
- 50       (b) a DNA which is hybridizable with a nucleotide sequence comprising the sequence of the nucleotide numbers 764 to 2380 in SEQ ID NO: 1 or a probe that can be prepared from the sequence under a stringent condition and encodes a protein having a glucose dehydrogenase activity.
26. A recombinant vector comprising the DNA according to claim 24 or 25.
- 55     27. The recombinant vector according to claim 26, which comprises nucleotide sequences encoding the signal peptide according to claim 18 and a β-subunit.

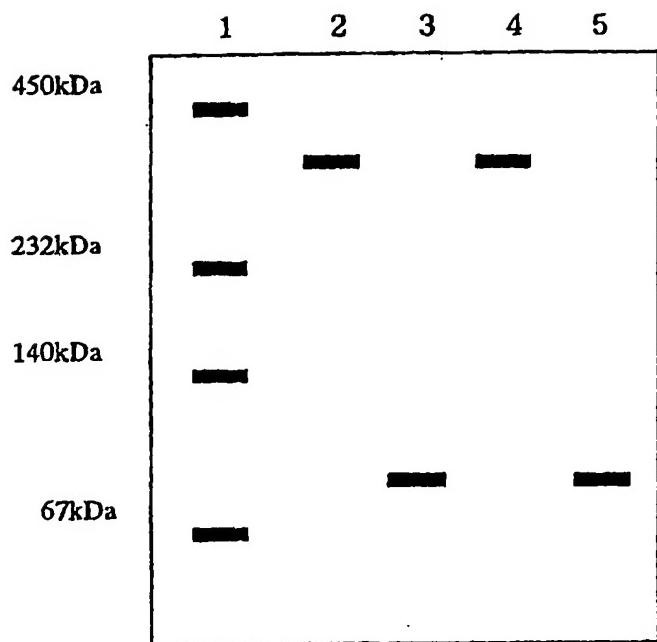
28. A transformant transformed with the DNA according to claim 24 or 25 or the recombinant vector according to claim 26 or 27.
- 5 29. A method for producing glucose dehydrogenase comprising the steps of culturing the transformant according to claim 28 to produce glucose dehydrogenase as an expression product of the DNA, and collecting it.
30. A *Burkholderia cepacia* KS1 strain (FERM BP-7306).
- 10 31. A glucose sensor using an enzyme electrode including the glucose dehydrogenase according to any one of claims 6 to 14, the protein according to any one of claims 20 to 23, the transformant according to claim 27 or the strain according to claim 30.
- 15 32. A glucose assay kit including the glucose dehydrogenase according to any one of claims 6 to 14 or the protein according to any one of claims 20 to 23.
33. A protein having the amino acid sequence of SEQ ID NO: 2.
34. A DNA encoding a protein having the amino acid sequence of SEQ ID NO: 2.
- 20 35. The DNA according to claim 34, which comprises the nucleotide sequence of the nucleotide numbers 258 to 761 in the nucleotide sequence of SEQ ID NO: 1.
36. A DNA comprising the DNA according to claim 34 or 35 and the DNA according to claim 24 or 25 in this order.
- 25 37. The DNA according to claim 36, which comprises the nucleotide sequence of the nucleotide numbers 258 to 2380 in the nucleotide sequence of SEQ ID NO: 1.
38. A recombinant vector comprising the DNA according to claim 36 or 37.
- 30 39. The recombinant vector according to claim 38, which comprises nucleotide sequences encoding the signal peptide according to claim 18 and a  $\beta$ -subunit.
40. A transformant transformed with the DNA according to claim 36 or 37 or the recombinant vector according to claim 38 or 39.
- 35 41. A method for producing glucose dehydrogenase comprising the steps of culturing the transformant according to claim 40 to produce glucose dehydrogenase as an expression substance of the DNA according to claim 36 or 37, and collecting it.

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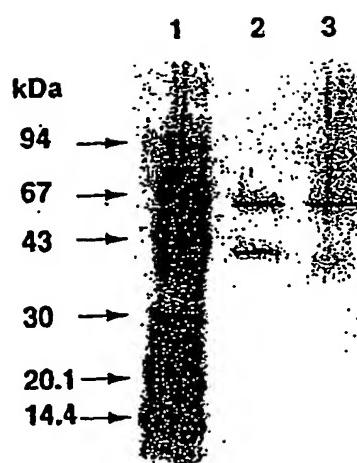
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*Fig. 1*



*Fig. 2*

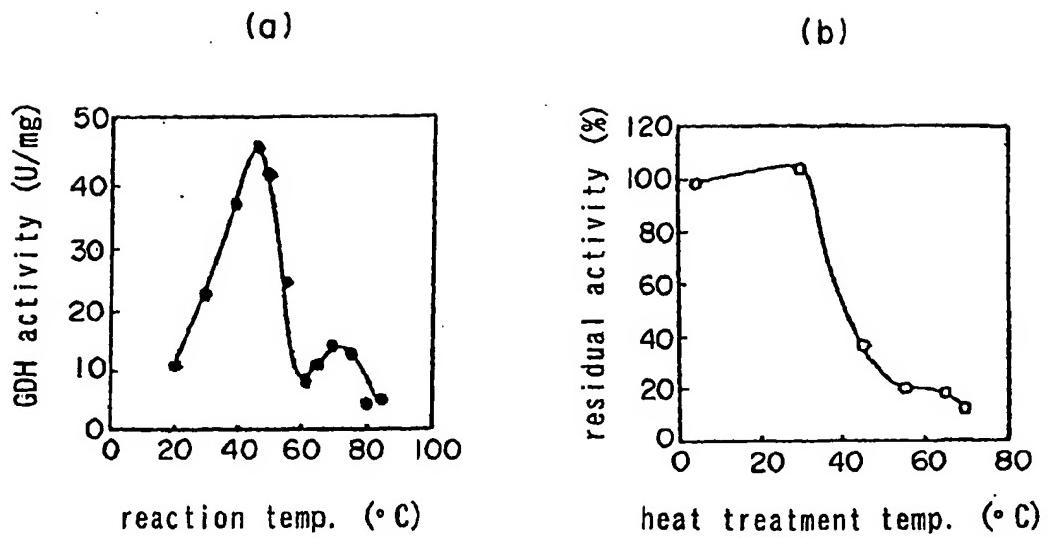


Fig. 3

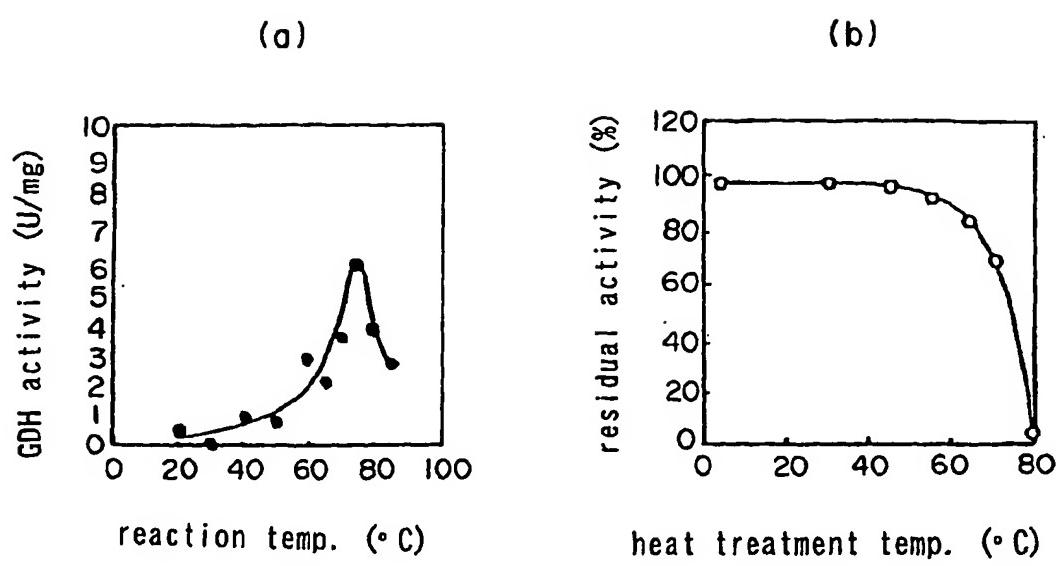


Fig. 4

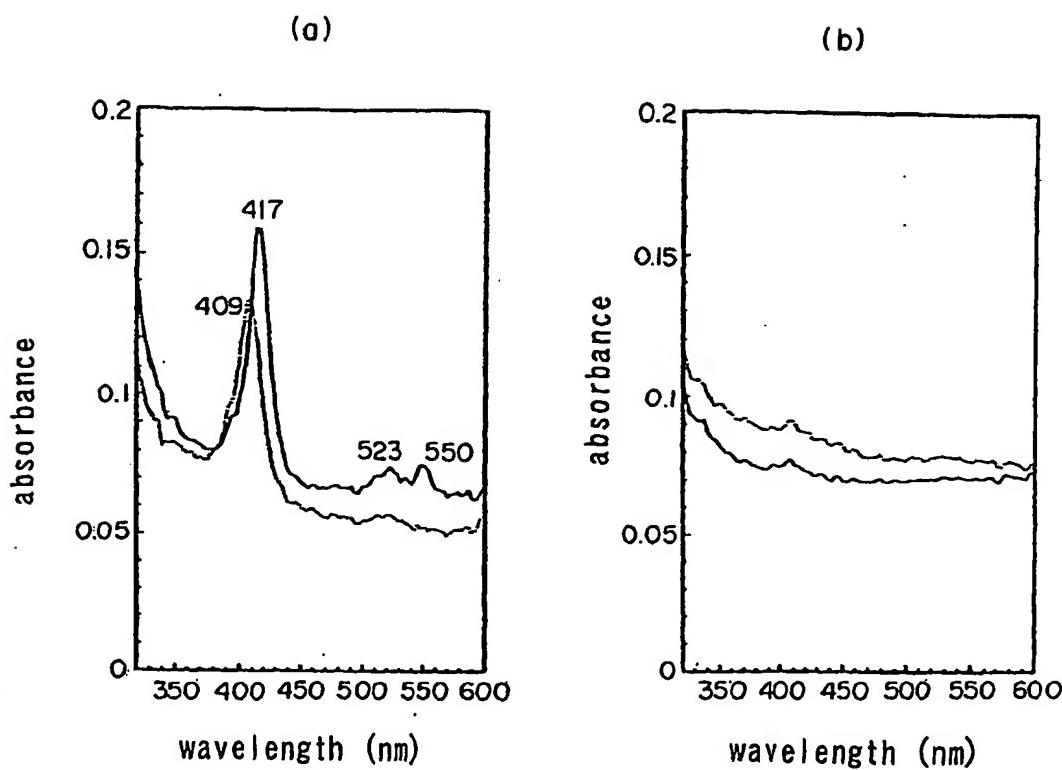


Fig. 5

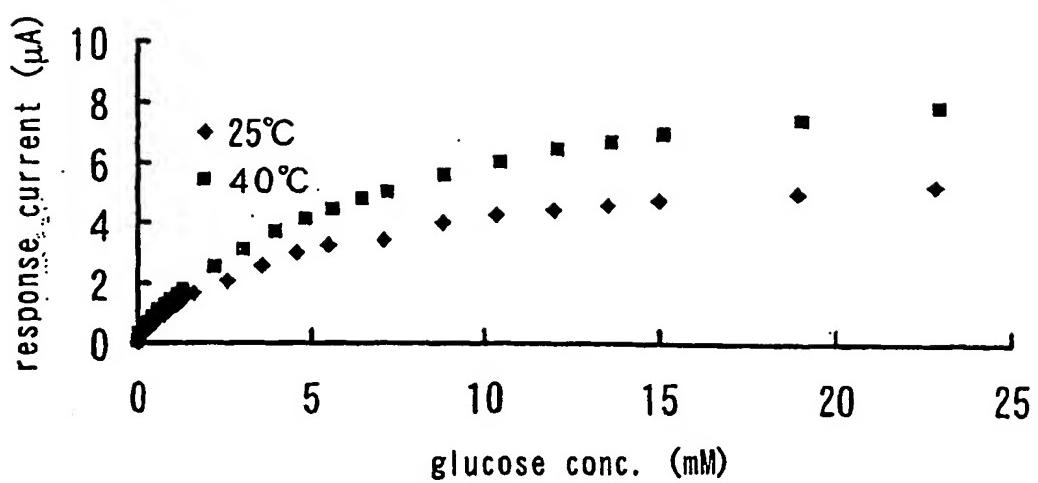


Fig. 6

INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP01/09556

A. CLASSIFICATION OF SUBJECT MATTER

Int.Cl' C12N15/53, C12N9/04, C12N1/21, C12N1/20, C12Q1/32

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int.Cl' C12N15/53, C12N9/04, C12N1/21, C12N1/20, C12Q1/32

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

JICST FILE (JOIS), WPI (DIALOG), BIOS (DIALOG),  
MEDLINE (STN), DDBJ/ Genbank/ EMBL/PIR/Swiss Prot/Geneseg

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	A. SAGE et al., Hoxose Phosphate Metabolism and Exopolysaccharide Formation in <i>Pseudomonas cepacia</i> , <i>Curr. Microbiol.</i> 1990, Vol. 20, No. 3, pages 191-198, table 1	1-14, 19-23, 30-33 15-18, 24-29
A	I. ESTANOL et al., Inorganic phosphate effect on alternate peripheral pathways of glucose catabolism in <i>Pseudomonas cepacia</i> , <i>FEMS Microbiol. Lett.</i> 1989, Vol. 60, No. 3, pages 295- 298, table 1	1-14, 19-23, 30-33 15-18, 24-29

Further documents are listed in the continuation of Box C.

See patent family annex.

"A"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance	"T"	Later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E"	earlier document but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search:  
22 January, 2002 (22.01.02)

Date of mailing of the international search report  
29 January, 2002 (29.01.02)

Name and mailing address of the ISA/  
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